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Fourteen-Day Inhalation Study in Rats, Using Aged and Diluted Sidestream Smoke from a Reference Cigarette

II. DNA Adducts and Alveolar Macrophage Cytogenetics

CHIN K. LEE, BUDDY G. BROWN, BETSY A. REED, CAROLYN A. RAHN, CHRISTOPHER R. E. COGGINS, DAVID J. DOOLITTLE, AND A. WALLACE HAYES

Research and Development, R. J. Reynolds Tobacco Co., Winston-Salem, North Carolina 27102

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The chemical constituents of cigarette smoke are greatly diluted in environmental tobacco smoke (ETS). In the typical indoor environment where cigarettes are smoked, the mean value of respirable suspended particles is approximately 0.1 mg/m^3. In this study, we used aged and diluted sidestream smoke (ADSS) of 1R4F University of Kentucky research cigarettes as a surrogate for ETS and exposed Sprague–Dawley rats nose-only to 0, 0.1, 1.0, and 10 mg wet total particulate matter (WTPM)/m^3 for 6 hr per day for 14 consecutive days. DNA from lung, heart, larynx, and liver was tested for adduct formation after 7 and 14 days of exposure and after 14 days of recovery. In addition, alveolar macrophages from animals exposed for 7 days were examined for chromosomal aberrations. Exposure-related DNA adducts were not observed in any of the animals at 0.1 or 1.0 mg WTPM/m^3, which represent ambient and 10-fold exaggerated ETS concentrations, respectively. Slight diagonal radioactive zones, characteristic of adducts observed in human smokers and in animals exposed to mainstream smoke, were observed, but only in lung and heart DNA of animals exposed to the highest concentration of ADSS (10 mg WTPM/m^3), a 100-fold exaggeration of typical field measurements of ETS. The mean relative adduct labeling values (±SE) were 8.7 (±0.2) adducts per 10^9 nucleotides for lung DNA and 5.7 (±0.7) adducts per 10^9 nucleotides for heart DNA after 14 days of exposure. No elevation in chromosomal aberrations was observed in alveolar macrophages. These results indicate a no-observed-effect-level (NOEL) of 1.0 mg/m^3 for DNA adduct formation in lung and heart and a NOEL of at least 10 mg/m^3 for the induction of chromosome aberrations in alveolar macrophages under the conditions of this study.

The biological significance of exposure to environmental tobacco smoke (ETS) has emerged as a subject of intense public discussion. ETS consists of sidestream smoke (SS), emitted from the burning end of a cigarette between puffs, and exhaled mainstream smoke (MS). Condensate from both MS (Doolittle *et al.*, 1990a; Lee *et al.*, 1990b) and SS (Doolittle *et al.*, 1990b) have been reported to be genotoxic *in vitro*. Several *in vitro* studies (Claxton *et al.*, 1989; Lewtas *et al.*, 1987; Ling *et al.*, 1987; Lofroth *et al.*, 1988; Lofroth and Lazardis, 1986) have reported that concentrated ETS particulate matter is mutagenic. Although cigarette smoke or its condensate is positive in many genotoxicity endpoints, its potency is very low compared to other complex mixtures to which humans are exposed, such as coke oven emissions, roofing tar, and diesel exhaust (Williams and Lewtas, 1985). Recently, Lewtas *et al.* (1991) estimated that the tumor-initiating potencies for the extractable organic matter from coke oven emissions and urban air are 1000- and 100-fold higher, respectively, than that of cigarette smoke condensate (CSC). They also concluded that the levels of DNA adduct formation with these complex mixtures correlated with tumor potency. In marked difference to concentrated smoke, constituents in ETS are highly diluted. Therefore, the critical question is not whether highly concentrated ETS is genotoxic *in vitro* but whether ETS, at its normal concentration range, is genotoxic *in vivo*.

The measurement of DNA adducts by the ^{32}P-postlabeling assay (Randerath *et al.*, 1981; Reddy and Randerath, 1986) is one of the best ways to assess DNA damage following exposure to complex mixtures. It is generally accepted that the majority of genotoxic chemicals are converted metabolically to electrophiles which attack nucleophilic centers in nucleic acids and proteins, resulting in the formation of covalent adducts (Miller and Miller, 1981; Hemminki, 1983). Therefore, DNA adducts serve as an indicator of exposure to genotoxic chemicals. The P_1 nuclease enhancement version of the ^{32}P-postlabeling assay (Reddy and Randerath, 1986) is sufficiently sensitive to detect one adduct per 10^{-9} to 10^{-10} nucleotides and therefore is ideally suited for complex mixtures of unknown chemical composition. Using this

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assay, several studies have reported DNA adducts in rodents following inhalation exposure to cigarette smoke (Gupta *et al.*, 1989; Bond *et al.*, 1989; Gairola and Gupta, 1991) as well as after skin painting with CSC (Randerath *et al.*, 1988; Reddy and Randerath, 1990). Adduct assays have been extended to include other complex mixtures such as diesel exhaust (Wong *et al.*, 1986; Bond *et al.*, 1989, 1990; Gallagher *et al.*, 1990), coal soot (Gallagher *et al.*, 1990), and lubricating oil (Schoket *et al.*, 1989). However, studies on the formation of DNA adducts in experimental animals after cigarette smoke exposure at or near the concentrations found in ETS have not been reported.

Recently, Rithidech *et al.* (1989) reported that pulmonary alveolar macrophages (PAM) of rats exposed to mainstream cigarette smoke developed chromosomal aberrations, thus demonstrating the usefulness of these cells as a tool for studying the cytogenetic effects of cigarette smoke in the rat lung.

In the present study, aged and diluted sidestream smoke (ADSS) was used as a surrogate for ETS. Cytogenetic damage in PAM and covalent DNA adducts in several internal organs of rats were assessed following exposure to different concentrations of ADSS for 14 consecutive days, 6 hr/day. The results demonstrate that chromosomes in PAM are not damaged and that adducts are formed only in the lungs and heart of animals exposed to the highest concentration of ADSS (10 mg WTPM/m^3). DNA adducts were not detectable at 0.1 mg WTPM/m^3, a typical concentration of human ETS exposure, or at a 10-fold higher concentration of 1.0 mg WTPM/m^3.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from New England Nuclear (Wilmington, DE). Micrococcal nuclease (100–200 units/mg) and nuclease P_1 (255 units/mg) were obtained from Sigma (St. Louis, MO). Calf spleen phosphodiesterase (2 units/mg) was obtained from Boehringer–Mannheim (Indianapolis, IN). Polyethyleneimine (PEI)-cellulose-coated thin-layer chromatographic (TLC) sheets (Machery Nagel) were obtained from Brinkmann Scientific (Westbury, NY). T_4 polynucleotide kinase was obtained from GIBCO–BRL (Gaithersburg, MD).

Experimental animals. Experimental animals have been described elsewhere (Coggins *et al.*, 1992). Briefly, 5-week-old Sprague–Dawley rats (Charles River, Raleigh, NC) were acclimated for 2 weeks prior to exposure in 2-cubic-meter stainless-steel inhalation chambers. The animal rooms had controlled lighting (12 hr dark and 12 hr light), temperature (20–24°C), and humidity (40–60% relative humidity). Animals were allowed unrestricted access to feed (Purina Rodent Chow 5002) and distilled water, except during the smoke exposures.

Smoke generation and exposure conditions. Smoke generation and exposure conditions have been described (Coggins *et al.*, 1992). Briefly, smoke was generated from 1R4F research cigarettes with a 30-port AMESA generator (CH Technologies, Westwood, NJ) fitted with an aluminum cone for collection of sidestream smoke. Cigarettes were smoked according to the Federal Trade Commission method (a 35-ml puff of 2 sec duration, once/min) except that instead of a fixed butt length, a fixed number of puffs (seven) was taken (Baumgartner and Coggins, 1980; Ayres *et al.*, 1990). Mainstream smoke was exhausted and discarded and the sidestream smoke was drawn into a common plenum. Different amounts of sidestream smoke were provided for each chamber and mixed with dilution air drawn from the animal room through HEPA filters. Target concentrations for suspended particulates were 0, 0.1, 1, and 10 mg/m^3. Exposures were for 6 hr per day, for 14 consecutive days.

Experimental design. For the DNA adduct assay, rats were divided into four groups: sham, low (0.1 mg WTPM/m^3), medium (1.0 mg WTPM/m^3), and high exposures (10.0 mg WTPM/m^3). Each group contained 12 male animals. Four animals in each group were killed after 7 and 14 days of exposure for DNA adduct analysis. The remaining 4 animals in each group were kept for a further 14 days without smoke exposure for the reversibility study.

DNA isolation and digestion. Whole lung, heart, liver, and larynx tissues were weighed and homogenized in Hanks' balanced salt solution (HBSS, Ca^{2+}- and Mg^{2+}-free). Homogenates were centrifuged and the supernatant extracts discarded. Pellets were resuspended in HBSS. DNA was isolated on a Genepure 341 nucleic acid purification system (Applied Biosystems, Inc., Foster City, CA) by solvent extraction and enzymatic digestion of protein and RNA. DNA concentration was estimated spectrophotometrically (1 A_{260} = 50 µg DNA/ml). Absorbance ratios (260/280) of all DNA samples ranged from 1.6 to 1.8. Samples of DNA were digested essentially as described by Gupta *et al.* (1982) with minor modifications. Five to 10 µg of DNA was digested for 3.5 hr at 37°C in a total volume of 10 µl containing 0.6 U of micrococcal nuclease and 5.0 µg of spleen phosphodiesterase (dialyzed 24 hr against water) in 100 mM $CaCl_2$ and 200 mM sodium succinate buffer, pH 6.0.

^{32}P-postlabeling assay. The nuclease P_1 procedure (Reddy and Randerath, 1986) was used as described with slight modifications. DNA digest samples were incubated for 45 min with nuclease P_1 under the following conditions: to 10 µl of DNA digest was added 4 µl of a solution containing each of the following: 2.0 µl of nuclease P_1 (4 µg/µl), 0.7 µl of 1 mM $ZnCl_2$, 0.7 µl of 0.4 M sodium acetate, pH 5.0, and 0.6 µl of distilled, deionized water. Immediately following nuclease P_1 treatment, 2.0 µl of 10 mM Ches–NaOH, pH 9.6, was added. DNA digests then were reacted with 75 µCi of [γ-^{32}P]ATP using 4.5 U of T_4 polynucleotide kinase and kinase buffer (200 mM Ches–NaOH, 100 mM $MgCl_2$, 100 mM dithiothreitol, and 10 mM spermidine, pH 9.6). Separation of the ^{32}P-labeled adducts was on PEI-cellulose TLC sheets using the following solvents: D1, 1.0 M sodium phosphate, pH 6.0 (overnight onto a wick); D3, 5.3 M lithium formate, 8.5 M urea, pH 3.5; D4, 1.2 M lithium chloride, 8.5 M urea, 0.5 M Tris base, pH 8.0 (onto a wick); D5, 1.7 M sodium phosphate, pH 6.0 (overnight onto a wick).

Quantification of DNA adducts. For each batch of [^{32}P]ATP, the specific activity was determined by measuring the kinase-catalyzed incorporated of radioactivity into 10 pmol of 2'-deoxyadenosine 3'-monophosphate (Reddy and Randerath, 1986). Values of specific activity fell within the range of 1.5 to 2.5 × 10^6 CPM/pmol. TLC maps were wrapped in Mylar plastic and scanned for 8 hr using the AMBIS radioanalytic imaging system (AMBIS Systems, Inc., San Diego, CA) under an argon atmosphere. A 3.2 × 3.2-mm resolution plate was used. The counting efficiency averaged approximately 20%. The AMBIS radioanalytic imaging system is a computer-controlled imaging system which directly quantifies radiation. Its use in ^{32}P-postlabeling assay of DNA adducts has been reported (Turteltaub *et al.*, 1990). The system has a two-dimensional proportional detector composed of 952 elements that simultaneously detect multiple β emissions. Sample patterns are replicated in the resulting computer picture which is displayed as a high-resolution image on a video monitor. Results are stored in permanent data files. Computer-generated images of the adduct maps were marked and scored for radioactivity. Background from the adjacent area was subtracted. The amount of adducts present in each sample was calculated from the radioactivity present in adduct spots or zones and the specific activity of the [γ-^{32}P]ATP used, and expressed as total number of adducts/10^9 nucleotides. In our experience, the images produced on the monitor equal, and often exceed, those of conventional autoradiography. The relative adduct labeling (RAL) values calculated from AMBIS-generated data were compared with those obtained by autoradiography and liquid scintillation counting. Results were comparable.

In vitro modification of DNA with CSC. One milligram of lung DNA isolated from Sprague–Dawley rats was treated with 5 mg (in 500 µl of DMSO) of CSC from Kentucky Reference 1R4F cigarettes in the presence of an S9 metabolic activation system. The S9 liver homogenate (obtained from Molecular Toxicology, Annapolis, MD) was prepared according to Ames *et al.* (1975). The S9 concentration in the mix was 5% (v/v) and 5.0 ml of the S9 mix was added to the reaction tube. The reaction mixture was incubated for 5 hr at 37°C. DNA was extracted with the ABI Genepure extractor.

In vivo modification of DNA with benzo[a]pyrene. Female B6C3F1 mice were given a single ip dose of 40 or 60 mg/kg of benzo[*a*]pyrene B[*a*]P in 0.1 ml of DMSO-corn oil (50/50 v/v). Livers were collected 43 hr after B[*a*]P administration. DNA was extracted by the A.S.A.P. genomic DNA isolation kit (Boehringer-Mannheim).

Pulmonary alveolar macrophage isolation and analysis of chromosomal aberration. Immediatley following the seventh consecutive daily ADSS exposure period, animals were injected ip with colchicine (6 mg/kg, 12 mg/ml colchicine stock solution). Four hours later, the animals were killed by asphyxiation with 70% carbon dioxide (CO_2) and the tracheas were cannulated with 14G iv catheters. The lungs and heart were removed as a block and placed in ice cold HBSS without Mg^{2+} or Ca^{2+} for 30 to 60 min. The lungs were lavaged repeatedly through the cannula using a 10-ml syringe filled with 7 ml of ice-cold HBSS. After the lungs were filled, they were massaged for 1 min and the lavage fluid was removed and placed in a 50-ml centrifuge tube on ice. Lavaging and massaging continued until approximately 50 ml of lavage fluid was collected. The lavage fluid was centrifuged and the supernatant fraction drawn off. The pellet was resuspended in prewarmed (37°C) 75 mM KCl and incubated at 37°C for 25 min. After centrifugation, the cells were fixed first with 6:1 absolute MeOH:glacial acetic acid and then three more times with Carnoy's fixative (3:1 absolute MeOH: glacial acetic acid). Metaphase spreads were prepared. Two slides per animal were stained for 7 min with Giemsa diluted 1:20 (10 ml Giemsa, 190 ml distilled H_2O) and mounted with coverslips using Depex mounting medium.

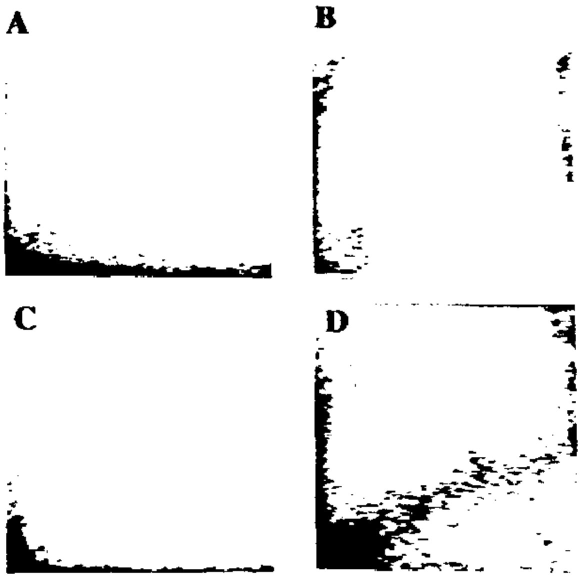

FIG. 1. Comparison of TLC maps of lung DNA from Sprague–Dawley rats exposed to aged and diluted sidestream smoke for 14 days: (A) sham exposure; (B) low exposure (0.1 mg WTPM/m³); (C) medium exposure (1.0 mg WTPM/m³); (D) high exposure (10 mg WTPM/m³). TLCs were scanned for 8 hr with AMBIS radioanalytic imaging system.

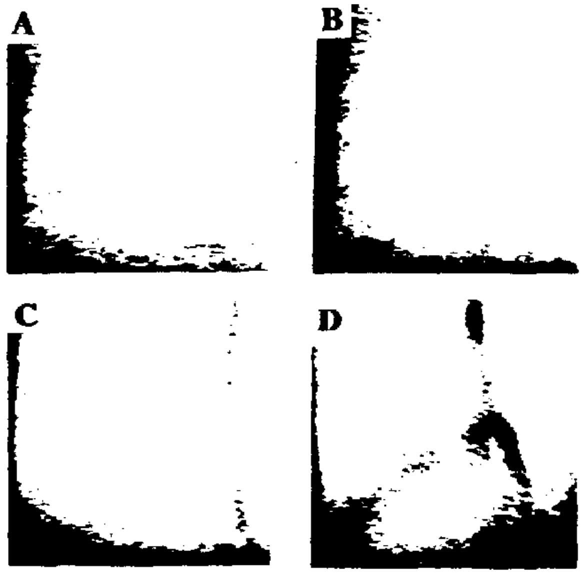

FIG. 2. Comparison of TLC maps of heart DNA from Sprague–Dawley rats exposed to aged and diluted sidestream smoke for 14 days: (A) sham exposure; (B) low exposure (0.1 mg WTPM/m³); (C) medium exposure (1.0 mg WTPM/m³); (D) high exposure (10 mg WTPM/m³). TLCs were scanned for 8 hr with AMBIS radioanalytic imaging system.

Fifty metaphases for each animal were scored for chromosome aberrations where possible. Sometimes 50 scorable cells were not available. Scarcity of scorable cells is not a sign of toxicity, but is due to the very low mitotic index of PAM (Pinkett *et al.*, 1966; Evans and Bills, 1969). Scoring was done without knowledge of treatment group.

Statistical analysis. Statistical significance ($p < 0.05$) was evaluated by analysis of variance for DNA adduct data and by Fisher's exact test for chromosomal aberration data.

RESULTS

After 14 exposure days, no overt signs of toxicity or significant body weight differences were observed between any of the exposed groups and the sham controls. Exposed lungs were not pigmented and the only histopathological changes observed were mild hyperplasia and inflammation of the nasal cavity in the high-exposure group only (Coggins *et al.*, 1992). These changes were reversible.

^{32}P maps of DNA samples from lung and heart tissues from animals in the highest exposure group (10 mg WTPM/m³) exhibited slight diffuse diagonal radioactive zones (DRZ) extending from close to the origin into the center of the chromatogram. Lung DNA from animals in the highest exposure group (10 mg WTPM/m³) exhibited DRZ after 7 and 14 days of exposure and the DRZ was still present after a 14-day recovery period. Heart DNA also exhibited faint but recognizable DRZs in the highest exposure group but only after 14 days of exposure and after the 14-day recovery period. Adduct maps of lung and heart DNA from the four

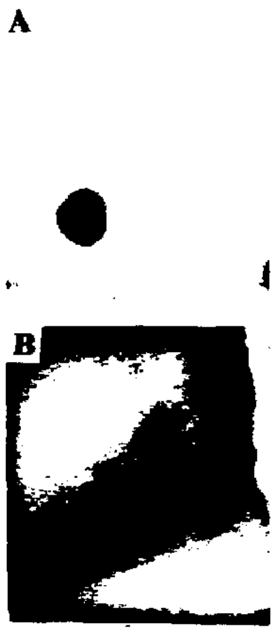

FIG. 3. TLC maps of B[*a*]P–DNA adduct from liver of mouse injected ip with 40 mg/kg of B[*a*]P (A) and CSC-DNA adduct prepared *in vitro* (B). TLCs were scanned for 8 hr with AMBIS radioanalytic imaging system. The RAL values are 839 adducts per 10^9 nucleotides for B[*a*]P–DNA adducts and 55 adducts per 10^9 nucleotides for CSC-DNA adducts.

exposure groups are shown in Figs. 1 and 2, respectively. The liver and larynx did not exhibit exposure-related adducts at any concentration or time point. Maps of CSC-DNA adducts from *in vitro* reactions and B[*a*]P–DNA adducts from liver of mice injected ip with B[*a*]P are shown in Fig. 3. Mean RAL values of the four organs (lung, heart, liver, and larynx) after 14 days of exposure are presented in Fig. 4.

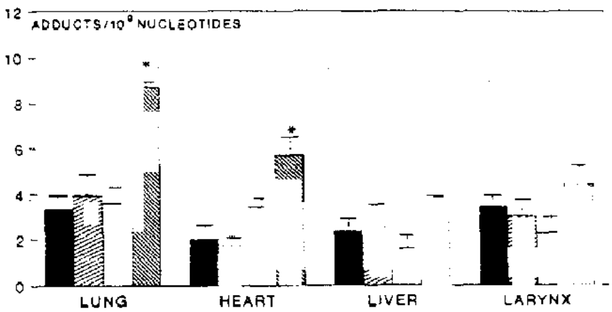

FIG. 4. RAL values (mean ± SE, $n = 4$) of DNA adducts in tissues of Sprague–Dawley rats after a 14-day exposure to a high concentration (10 mg WTPM/m^3) of aged and diluted sidestream smoke. Differences between the groups were tested by ANOVA. *Significantly different ($p < 0.05$) from sham control. (■) Sham; (□) low, 0.1 mg WTPM/m^3; (□) medium, 1 mg WTPM/m^3; (□) high, 10 mg WTPM/m^3.

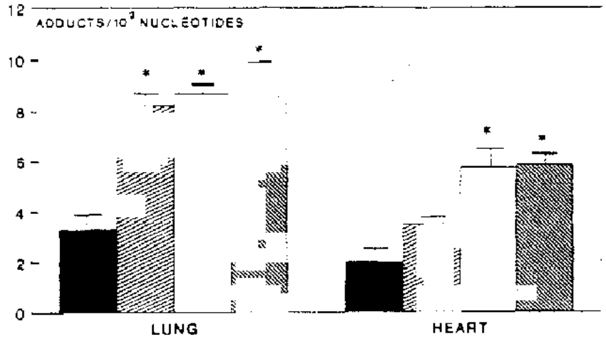

FIG. 5. Comparison of RAL values (mean ± SE, $n = 4$) of DNA adducts in lung and heart after 7- and 14-day exposures to high concentration (10 mg WTPM/m^3) of aged and diluted sidestream smoke and after a 14-day recovery period. Differences between the groups were tested by ANOVA. *Significantly different ($p < 0.05$) from sham control. (■) Sham, (□) 7 days, (□) 14 days, (□) 14-day recovery.

Only the lungs and heart from the highest smoke exposure (10 mg/m^3) contained adduct concentrations significantly higher than sham controls. The RAL values were 7 to 10 adducts per 10^9 nucleotides (21 to 30 amol/μg DNA) for lung and 4 to 7 adducts per 10^9 nucleotides (12 to 21 amol/μg DNA) for heart. In both lung and heart, the amount of adducts was similar at the end of the 14-day exposure period and after the 14-day reversibility period (Fig. 5).

The results of the chromosomal aberration assay in PAM after 7 consecutive exposure days are presented in Table 1. Positive control animals injected with cyclophosphamide exhibited a statistically significant increase in chromosomally aberrant cells; however, no statistically significant increase in cells with aberrations was observed in smoke-exposed animals.

TABLE 1
Chromosome Aberration Analysis in Pulmonary Alveolar Macrophages of Rats Exposed to Aged and Diluted Sidestream Smoke for 7 Days

Treatment	Number of animals	Number of cells analyzed	Number of aberrant cells	Percentage cells with aberrations
Sham exposure	3	150	2	1.3
Low exposure (0.1 mg WTPM/m^3)	4	189	2	1.1
Medium exposure (1.0 mg WTPM/m^3)	3	77	0	0
High exposure (10 mg WTPM/m^3)	3	150	6	4.0
Cyclophosphamide (10 mg/kg)	2	89	12	13.5*
Saline (0.5 ml/kg)	3	150	1	0.7

* Statistically significant ($P < 0.05$).

DISCUSSION

The P_1 nuclease enhancement version of the ^{32}P-postlabeling assay (Reddy and Randerath, 1986) is the most sensitive method available for evaluating DNA adducts following *in vivo* exposure to complex mixtures. DNA adducts have been observed in experimental animals and humans following exposure to a variety of complex mixtures including mainstream cigarette smoke. For example, the presence of DNA adducts in smokers has been reported, with significantly lower levels in ex-smokers and nonsmokers (Phillips *et al.*, 1988, 1990; Randerath *et al.*, 1989; Cuzick *et al.*, 1990). In rodents, adduct formation has been reported in lungs and other respiratory tissues (Bond *et al.*, 1989; Gupta *et al.*, 1989; Gairola and Gupta, 1991) following smoke exposure. On the basis of these reports, the rat appears to be a suitable animal model for molecular dosimetry of covalent DNA binding at low concentrations of complex mixtures such as ETS.

DNA adducts generally decrease and eventually disappear upon cessation of exposure to genotoxic agents. Adducts in ex-smokers are reported to be significantly less than in current smokers and eventually reach the levels of nonsmokers (Phillips *et al.*, 1988; Randerath *et al.*, 1989). In baboons exposed to cigarette smoke, DNA adducts disappeared with time during smoking cessation (Marshall *et al.*, 1991). However, the rate of decrease may depend on the tissues involved. Slower disappearance of polycyclic aromatic hydrocarbon–DNA adducts in lung compared to skin has been reported (Schoket *et al.*, 1989). In the present study, 2 weeks were insufficient to show a complete disappearance of the adducts in either lung or heart from the high-exposure animals. This finding is consistent with observations reported by others. A decrease in adduct levels was not observed until 4 weeks after the cessation of diesel exhaust exposure to rats (Bond *et al.*, 1990). Lung DNA adducts from cigarette-smoke-exposed rats remained at the same level 1 week after cessation of exposure with a significant decrease after 19 weeks (Gupta *et al.*, 1989).

Rithidech *et al.* (1989) exposed animals to approximately 100 to 200 mg TPM/m^3 for 6 hr/day, 5 days/week for 22 to 24 days and reported an increase in the frequencies of chromosomal aberration in exposed animals. Other cytogenetic studies on smoke-exposed rats (Lee *et al.*, 1990a; Basler, 1982) and hamsters (Korte *et al.*, 1981), however, failed to induce chromosome aberration, sister chromatid exchanges, or micronuclei formation in bone marrow and lymphocytes. Based on these results, PAM appears to be a more sensitive tissue for measuring the clastogenic potential of inhaled tobacco smoke than is bone marrow. In our study, none of the exposed animals, including the highest exposure group, yielded PAM with increased chromosome aberration.

Concentrations of ETS in indoor environments vary, but average concentrations of respirable suspended particles are approximately 0.1 mg/m^3 (Oldaker, 1989; Oldaker *et al.*, 1990a,b). A weakly visible DRZ in lung and heart tissues was observed in rats after exposure to ADSS, but only at the highest exposure (10 mg WTPM/m^3). Although smoke-related DNA adducts have been reported in the larynx in human smokers (Randerath *et al.*, 1989) and in rats exposed to mainstream smoke (Gairola and Gupta, 1991), our study did not detect DNA adducts in this organ or liver at the high exposure (10 mg WTPM/m^3), a 100-fold exaggerated ETS concentration. It is significant that concentrations (0.1 and 1.0 mg WTPM/m^3), which represent an average level of ETS in most places where smoking is allowed and a 10-fold increase in concentration, respectively, did not result in any exposure-related adducts. Even in tissues with visible DRZ, the level of adducts was near the detection limit. None of the concentrations tested significantly increased chromosome aberrations in PAM. Thus, under the conditions of these studies, 1.0 mg WTPM/m^3 represents a no-observed-effect-level (NOEL) for DNA adduct formation in lung and heart and a NOEL of at least 10 mg/m^3 exists for the induction of chromosome aberrations in alveolar macrophages.

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